Impact of Benzene Metabolites on Differentiation of Bone Marrow Progenitor Cells

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Interleukin-3 (IL-3) and granulocyte/macrophage–colony-stimulating factor (GM–CSF) are responsible for maintaining survival and stimulating growth of early dormant hematopoietic progenitor cells (HPC). These cytokines exhibit extensive overlap, with GM–CSF supporting growth and differentiation of myeloid HPC. A characteristic shared by a diverse group of leukemogens is the ability to act synergistically with GM–CSF to increase clonogenic response. Previous studies have revealed that pretreatment of murine HPC with hydroquinone (HQ) but not phenol, catechol, or trans-trans-muconaldehyde results in a selective enhancement of GM–CSF but not IL-3-mediated clonogenic response. Pretreatment of murine bone marrow cells with these agents or their metabolites in vitro results in increased numbers of HPC dividing and forming colonies in response to GM–CSF but not IL-3. The present studies explored the molecular mechanisms associated with altered cytokine response in early HPC in murine bone marrow and extended our initial observations in murine bone marrow to human bone marrow cells. HQ pretreatment of murine HPC did not induce either an up- or a down-regulation of GM–CSF receptors or any change in receptor affinity. CD34+ cells, which represent between 1 and 5% of human bone marrow, contain virtually all clonogenic stem and HPC. Pretreatment of CD34+ cells (~95% purity) with HQ also results in enhanced clonogenic response with GM–CSF but not IL-3. These findings suggest that an early step in chemical leukemogenesis may involve transient alterations in the regulation of cytokine response to GM–CSF. — Environ Health Perspect 104(Suppl 6):1247–1250 (1996)

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Introduction

A variety of normal tissues are characterized by high rates of cell turnover in which mature cells are consumed or die. These cells are continually replaced by others that are derived from primitive, undifferentiated cells. Cell division represents the most important inherent source of increased risk of mutation and chromosomal aberrations throughout life, and a number of agents whose major biological effect is to induce cell proliferation are major risk factors for specific human cancers (1). Therefore, it is not surprising that cell maturation and proliferation in such tissues is highly ordered so that replication of cells with the greatest potential for neoplastic transformation is tightly controlled, and so that cells undergoing the most rapid turnover are committed to terminal differentiation or apoptosis (2). Normal hematopoiesis is probably the ultimate example of a tightly regulated process in which cell survival, differentiation, and proliferation are inextricably linked and controlled by multiple growth factors or cytokines that collaborate to regulate hematopoiesis (3).

The results of a number of studies utilizing a variety of different systems support the observation that altered response to hematopoietic cytokines, specifically granulocyte/macrophage–colony-stimulating factor (GM–CSF) or interleukin-3 (IL-3), feature prominently in early events in the leukemogenic process. Enhanced expression of GM–CSF or IL-3 results in profound myelodysplastic changes (4–6), and altered clonogenic response to GM–CSF is a frequent early observation in the development of acute myelogenous leukemia (AML) (7,8). Moreover, inhalation exposure of mice to benzene enhances clonogenic response to GM–CSF (9,10), and chronic exposure to high concentrations induces a persistent myeloproliferative disorder (10,11). These specific alterations are most likely due to hydroquinone (HQ), which selectively enhances clonogenic response to GM–CSF in murine bone marrow cells (12,13). Our work has focused on understanding the relationship between altered response of primitive bone marrow cells to cytokines involved in proliferation and survival and early events in leukemogenesis. These studies have revealed that the potential to act synergistically with GM–CSF to induce a clonogenic response in primitive HPC is a characteristic shared by a variety of leukemogenic agents (12,13). Pretreatment of murine bone marrow cells with these agents or their metabolites in vitro results in increased numbers of HPC dividing and forming colonies in response to GM–CSF but not IL-3. The present studies were designed to evaluate potential molecular mechanisms associated with altered cytokine response in early HPC in murine bone marrow and to extend our initial observations in mice to human bone marrow cells.

Materials and Methods

Reagents. Murine recombinant (r)GM–CSF (5×10^7 U/mg) and human rGM–CSF (1.25×10^7 U/mg), recombinant stem cell factor (SCF) (10^5 U/mg), and rIL-3 (2×10^7 U/mg) were generous gifts from Immunex (Seattle, WA). Modified Iscove's medium, RPMI-1640, L-glutamine, penicillin/streptomycin solution, and phosphate-buffered saline (PBS) were purchased from Life Technology (Grand Island, NY). Fetal bovine serum (FBS) was supplied by Gemini Bioproducts (Calabasas, CA).

Lympholyte-M was obtained from Accurate Scientific (Westbury, NY). Histopaque-1077, methyl cellulose, bovine serum albumin (BSA), HQ, 2-mercaptoethanol, dibutyl phthalate, and bis(2-ethylhexyl)phthalate were purchased from Sigma Chemical Company (St. Louis, MO). The MiniMACS separation system and CD34 cell isolation kit were supplied by Miltenyi Biotech Inc. (Sunnyvale, CA). Fluorescein isothiocyanate (FITC) conjugated anti-CD34 (HPCA-2) was purchased from Becton Dickinson (San Jose, CA). Enzymobead radioiodination reagent was obtained from Bio-Rad (Melville, NY). I^{125} was purchased from Amersham (Arlington Heights, IL).

Animals. Four-week-old male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were acclimated for 2 weeks prior to use and were housed 10 to a cage in sterile chambers with filter tops. Mice were allowed autoclaved food (3000, Agway, Syracuse, NY) and sterilized water *ad libitum*. All procedures performed on mice were approved by the University of Colorado Health Sciences Center's Animal Care and Use Committee.

Human Bone Marrow. Human bone marrow was obtained with informed consent from normal adult volunteers by aspiration from the posterior iliac crest. These studies were conducted under a protocol approved by the University of Colorado Health Sciences Center's Internal Review Board.

Mouse Bone Marrow Cell Preparation. Mononuclear nonadherent bone marrow cells were harvested from femora as previously described (*12*). Briefly, animals were killed by cervical dislocation and bone marrow was flushed from femora with PBS containing 1% BSA using a 5-ml syringe with a 22-gauge needle. A single cell suspension was obtained using a Pasteur pipet, which was then purified over Lympholyte-M. The recovered buffy layer was removed and washed twice in PBS/BSA. Nonadherent cells were obtained by incubating the cells at 2 × 10^6/ml in culture flasks for 1 hr at 37°C in PBS/BSA.

Human Bone Marrow Cell Preparation. Mononuclear cells were isolated by using Histopaque-1077 per manufacturer's instructions. CD34$^+$ cells were purified from the mononuclear cells by using the MiniMACS purification system according to manufacturer's instructions. The CD34 purity was determined by labeling the cells with FITC-conjugated anti-CD34 monoclonal antibody (HPCA-2) specific for a

CD34 epitope distinct from that used in the purification process (QBEND-10) followed by flow cytometry analysis (Epics 752, Coulter Electronics, Hialeah, FL).

Chemical Exposures. Cells were incubated with PBS or HQ dissolved in PBS for 30 min at 37°C, washed in Iscove's medium containing 10% FBS and used in the colony-forming units (CFU) assay.

CFU Assay. The CFU assay was performed as previously described (*12*). Briefly, bone marrow cells were plated in 35-mm culture dishes at a concentration of 1 to 5 × 10^4 cells/ml in 1 ml modified Iscove's medium containing 10 to 20% FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, 1.2% (wt/vol) methyl cellulose, and recombinant cytokines. Each cytokine was used at concentrations experimentally determined to produce maximal colony formation. All cultures were maintained at 37°C in 5% CO$_2$. Colonies consisting of 30 or more cells were scored on day 14. Five plates were scored for each *in vitro* treatment group and results expressed as the mean ± 1 SEM. Significant differences ($p \le 0.05$) between pretreated and control groups were determined using the Student's t-test. Statistics were calculated using Excel 4.0 (Microsoft Corp., Redmond, WA).

GM–CSF Receptor Binding. I^{125}-labeled murine GM–CSF was prepared using Enzymobead radioiodination reagent as modified by Park et al. (*14*). Binding assays were carried out using a phthalate oil separation method (*15*) as performed by Park et al. (*14*), and association/dissociation kinetics were analyzed as previously described (*16,17*). Specific activity of each preparation was determined by self-displacement analysis (*18*).

Results

Human HPC Purification

Representative sampling of murine bone marrow cells is simplified by the ability to evacuate and collect all the cells contained in the long bones of a mouse. Moreover, bone marrow samples obtained from syngeneic mice maintained under controlled conditions are, for all intents and purposes, identical. In contrast, human bone marrow samples are collected by aspiration of a relatively small volume from the iliac crest of normal human volunteers who are genetically unrelated. To assure as uniform and standardized preparations of human HPC as possible, an isolation procedure was utilized

based on magnetic bead gradient purification of CD34$^+$ cells (*19*). The CD34$^+$ cell population contains all clonogenic cells identified in human bone marrow, including both long-term and short-term repopulating cells (*20*) and is markedly enriched for primitive HPC. Using this method, the purity of CD34$^+$ routinely obtained from unrelated volunteers is greater than 91% and often approaches 99% (Table 1). The flow cytometric distribution of the CD34 marker in purified human bone marrow cells is illustrated in Figure 1.

Effects of HQ Pretreatment on Human Bone Marrow Cells

Similar to previously reported results in murine bone marrow, HPC normally unresponsive to rGM–CSF are induced by HQ pretreatment to selectively respond to human rGM–CSF and not human rIL-3 (Figure 2). HQ pretreatment at 10^{-6} M induces a 291% increase in clonal response to rGM–CSF. Similar to results obtained in mice, HQ pretreatment of human HPC does not mimic SCF, since cells grown in the presence of rGM–CSF + rSCF are also

Table 1. Purification of CD34$^+$ cells from human bone marrow.

Sample	% CD34$^+$	Yield, x 10E6	% of starting cells[a]
A5.0	96.5	1.05	1.6
A5.1	90.6	0.32	0.8
A5.2	93.3	1.20	2.8
A5.3	91.4	0.27	1.3
A5.4	94.2	2.55	8.3
A5.5	98.3	3.14	8.0
A5.6	97.6	0.80	3.1

[a]Percent of starting cells is determined by the number of purified CD34$^+$ cells divided by the starting number of cells labeled with anti-CD34 microbeads.
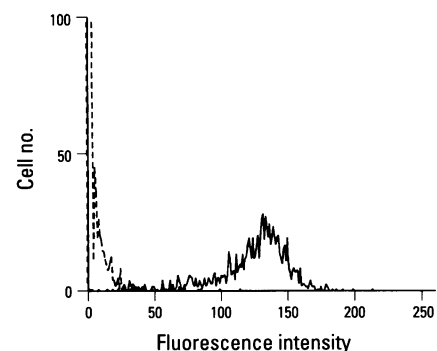

Figure 1. CD34 expression on Ficoll purified (---) and CD34 purified (—) human bone marrow. The purified cells are 98.05% positive for CD34.
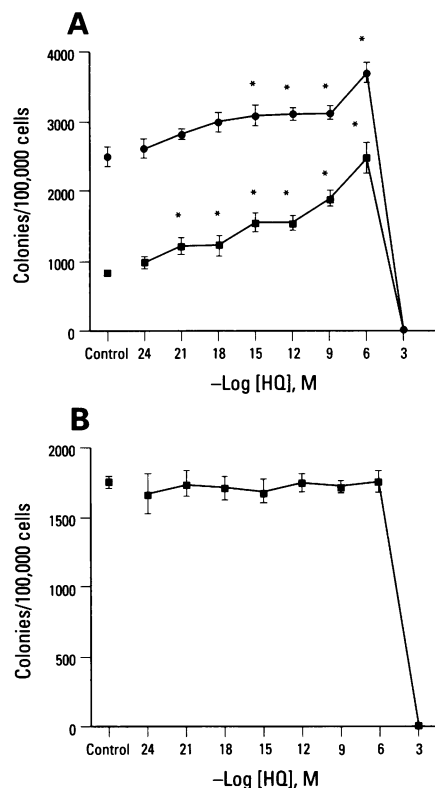
Figure 2. Effects of HQ pretreatment of CD34+ human bone marrow cells on recombinant (A) GM–CSF with or without SCF and (B) IL-3 stimulated colony formation. (A) ■, rGM–CSF at 5 ng/ml; ●, rGM–CSF at 5 ng/ml + rSCF at 25 ng/ml; (B) ■, IL-3 at 10 ng/ml. Error bars indicate 1 SEM for five cultures and are omitted when they are smaller than the symbol. Asterisk indicates significant increase compared to controls treated with PBS ($p \leq 0.05$).

recruited to respond by HQ pretreatment independent of rSCF.

Studies on the Role of Altered GM–CSF Receptor Expression

A series of experiments was conducted to evaluate potential mechanisms associated with HQ-induced enhancement of clonogenic response in murine bone marrow cells. Previous experiments utilizing murine HPC essentially depleted of stromal, and lineage-committed bone marrow cells suggest that the observed synergistic response to rGM–CSF represents an intrinsic effect on responding HPC and is independent of altered production of cytokines by accessory cell populations (*12,13*). Because HQ-induced synergism is specific for GM–CSF, the possibility that HQ pretreatment results in altered GM–CSF receptor expression was evaluated. We measured 280 high- and 1700
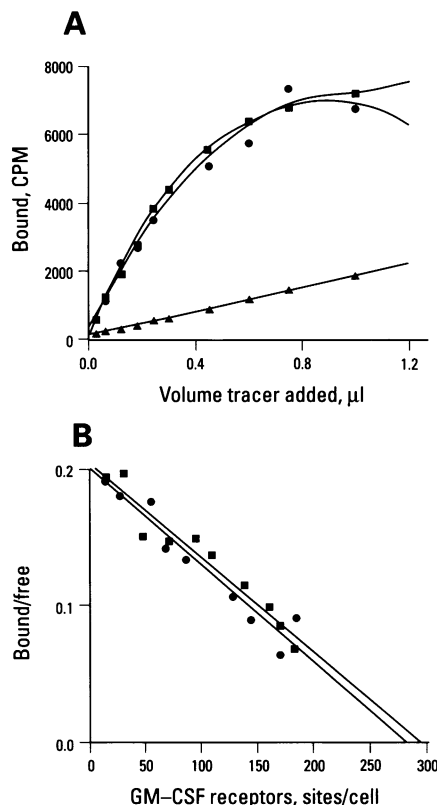

Figure 3. Binding isotherm (A) and Scatchard plot (B) of GM–CSF binding to HQ- and PBS-treated murine bone marrow cells.

low-affinity sites, respectively, for an enriched population of murine HPC that compares favorably with previously reported ranges for high- (10–100) and low-(300–500) affinity sites/cell in unfractionated bone marrow cells (*21*). Nonspecific binding occurred linearly with increasing concentration of ^{125}I-rGM–CSF and was in every case < 3% of the total disintegrations per minute added. The results of these experiments presented in Figure 3 indicate that HQ pretreatment has no detectable influence on high-affinity GM–CSF receptors, suggesting that the increased recruitment of GM–CSF responsive myeloid progenitor cells by HQ pretreatment is not the result of either an up- or a down-regulation of GM–CSF receptors or changes in receptor–ligand affinity.

Discussion

We have demonstrated that clonogenic response in selected populations of HPC is subject to chemical influence and that chemical/drug pretreatment can synergize with growth factors, resulting in clonogenic recruitment of subpopulations of murine

and human HPC (*12,13*). Hematopoiesis is characterized by a hierarchy of progenitor cell types with specific genetic repertoires and phenotypic characteristics that define their capacity with respect to survival, self-renewal proliferation, and differentiation. It is generally recognized that proliferation of committed multi- or single-lineage HPC is controlled by multiple growth factors with overlapping specificity, and a variety of positive and negative control mechanisms have been described that are required to maintain cell homeostasis in this system (*22*). It is likely that altered clonogenic response to cytokines involves alterations in molecular signals associated with cell survival, proliferation or differentiation. Nevertheless, it is not known whether the cellular transduction mechanism(s) governing these processes are subject to individual environmental manipulation. For HQ, synergism appears to be due to an intrinsic increase of additional clones of cells that are responsive to the growth factor rather than to changes in the rate of cell proliferation (*12*). The mechanism of chemical-induced synergism does not likely involve secondary cytokine production because *a*) synergism is highest in purified preparations of HPC, *b*) no colonies are formed in the absence of exogenous cytokine, and *c*) synergy is specific for GM–CSF. In contrast, comitogenic cytokines synergize with both IL-3 and GM–CSF and in certain cases granulocyte colony-stimulating factor and macrophage colony-stimulating factor. In the case of HQ, synergism is specific for GM–CSF and is optimal following pretreatment for as little as 30 min. À priori, these results suggest an event involving modulation of cytokine receptor expression: altered signal transduction or modulation of the fate (i.e., survival) of responding cells. Pretreatment of murine bone marrow cells with HQ under conditions that result in enhanced clonal response to rGM–CSF is not accompanied by alterations in GM–CSF receptor expression as measured by changes in either the number of affinity or surface receptor sites. Analogous experiments with monoclonal antibodies specific for IL-3 receptor-associated proteins induce effects similar to HQ (*23*). These antibodies produced no response alone, showed synergy with GM–CSF, and did not affect the IL-3 response of HPC. In studies using the same system and the 32Dc13 cell line, HQ did not show any capacity to generate a partial IL-3 signal (data not shown). In addition, using neutralizing antibodies to murine IL-3, we ruled out the possibility

that HQ is causing a release of IL-3 (data not shown). The most plausible explanation based on available data is that HQ activates a mechanism involving one or more secondary signals that are not sufficient to induce HPC into cycle but will synergize with GM–CSF to do so.

In a rapidly dividing tissue such as bone marrow in which control of stem and progenitor cell proliferation commands a high priority, changes in proliferation or survival may predispose susceptible target cells to replication-dependent damage and subsequent neoplastic transformation. Issues remaining to be addressed include the elucidation of the molecular mechanism(s) of enhanced cytokine response and clarification of the impact of increasing, by as much as 2-fold, the size of the replicating cell population that is potentially targeted in the development of AML. Characterization of the respective roles of proliferation and differentiation in the regulation of HPC will provide a biological basis for improving predictions of the risk of leukemogenesis secondary to benzene exposure.

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